*Malays. Appl. Biol. (2011) 40(1): 25-32*

# THE EFFECTS OF NEROLIDOL, ALLICIN AND BERENIL ON THE MORPHOLOGY OF *Trypanosoma evansi* IN MICE : A COMPARATIVE STUDY USING LIGHT AND ELECTRON MICROSCOPIC APPROACHES

MOHD-SHUKRI, H.B.[1]* & ZAINAL-ABIDIN, B.A.H.[2]*

*[1]Department of Basic Health Sciences, Kulliyyah of Allied Health Sciences,*
*International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia*
*[2]Faculty of Medicine, University Teknologi MARA (UiTM)*
*40450 Shah Alam, Selangor, Malaysia*
**E-mail : [1]vibrio_parahaemolyticus2000@yahoo.com and [2]zainala@ukm.my*

## ABSTRACT

Cell morphological changes are normally considered as the indirect evidence of the effect of test materials on targeted cells. In this study, the effects of nerolidol ($C_{12}H_{26}O$) and allicin ($C_3H_5SS(O)C_3H_5$), extracted from cardamon (*Eiettaria cardamomum*) and garlic (*Allium sativum*) respectively, were compared with the effect of berenil (standard anti-trypanosomal drug) on the morphological changes of a protozoan parasite *Trypanosoma evansi* in mice, determined by light microscope and electron microscopy. Groups of male ICR mice strain were subjected to infection with and without the parasite trypanomastigote ($5.0 \times 10^3$ *T. evansi* per mouse), treated with nerolidol and allicin, and treated with berenil or distilled water as the control. Blood samples were collected and prepared both for the observation under light and electron microscopes. Parasites observed at the trypomastogote stage had adverse morphological changes due to berenil treatment and after the $2^{nd}$ – $3^{rd}$ hour post-treatment, the parasites became stiffened and tapered at both ends and distorted with fractured flagella and loss of undulating membranes before totally disintegrated and cleared from the blood at the $6^{th}$ – $7^{th}$ hour post-treatment. The morphological changes in the nerolidol-treated group only appeared after the $23^{rd}$ day post-treatment and continued gradually until the $25^{th}$ day post-treatment when the parasites became stiff, lost their undulating membrane but the free flagella remained intact. Total disfigurement was only observed at the $27^{th}$ day post-treatment. On the other hand, parasites in the allicin-treated group also showed marked morphological changes, although not as profound as changes due to berenil. Changes started to occur only after the $18^{th}$ day post treatment, and gradually intensified up until the $90^{th}$ day post treatment although the treatments were terminated on day $30^{th}$. The parasite also became crescent in shape and lost their undulating membranes and cytoplasm where total disfigurement was only observed in the $95^{th}$ days post-infection. All mice in the negative group (untreated-infected) succumbed to infection with drastic increase of parasitaemia while all the infected and berenil-treated mice survived the infections for more than 100 days post-infection. These observations indicate that, to a certain extent, nerolidol and allicin showed convincing and promising anti-trypanosomatidal activity against the morphology of *T. evansi* in mice. Further studies are required to elucidate the mechanism(s) of action of these compounds.

**Key words:** nerolidol, allicin, berenil, *Trypanosoma evansi*

## INTRODUCTION

*Trypanosoma evansi* is the parasite that can cause trypanosomiasis or surra disease to most of the mammals such as goat, pig, camel, cow and donkey (Ventura *et al.*, 2000). Its wide range and chronic type of infections were believed to have affected the productivity of livestock especially in country where veterinary sectors play a major role as economic resources (Yabu, 1998). It was originally discovered in horses and camels by Griffith Evans in Punjab India and in Malaysia, it was first recorded in buffaloes, goats and dogs in 1903 (Zainal-Abidin, 1992). Nerolidol or 3,7,11-trimethyl-1,6,10-dodecatrien-3-ol ($C_{12}H_{26}O$) is an active compound that naturally occurs in oil form in cardamom seed, *Eiettaria cardamomum*. In South Asia, cardamom is widely used to treat infections in teeth, to prevent and treat sore throat, congestion of the lungs and pulmonary tuberculosis, inflammation of eyelids and

* To whom correspondence should be addressed.

also to treat digestive disorders (Ravindran, 2002). It was *in vitro* proven that nerolidol was able to inhibit the growth of promastigotes stage of *Leishmania amazonensis, L. braziliensis* and *L. chagasi* as well on the amastigotes stage of *L. amazonensis* with its 50 % inhibitory concentrations ($IC_{50}$) were 85 µM, 74 µM, 75 µM and 67 µM, respectively (Denise *et al.*, 2004). While, allicin or diallyl thiosulfinate ($C_3H_5SS(O)C_3H_5$), is the main biologically active compound derived from garlic, *Allium sativum* that has been used as a general food and a remedy for a long time. It was found that *Entamoeba histolytica* was very sensitive to allicin, and at 30 g/ml, allicin totally inhibited the growth of amoeba cultures (Ankri *et al.*, 1997). Allicin (30 µg/ml) was also very efficient to inhibit the growth of other protozoan parasites such as *Giardia lamblia*, *Leishmania major*, *Leptomonas colosoma* and *Crithidia fasciculate* (Rabinkov *et al.*, 1998). The present study was carried out to determine the anti-trypanosomal effects of nerolidol and allicin on the morphology of *T. evansi* in mice, determined via both light and electron microscopes.

## MATERIALS AND METHODS

Male ICR mice strain (*Mus musculus*) aged 6 – 8 weeks and 20 – 25 g body weights were used to maintain *T. evansi* stock and as experimental mice. All groups for treatment and control consisted of six mice per group and the inoculum used was $5.0 \times 10^3$ *T. evansi* per mouse and administrated intraperitoneally. The blood slides were prepared from mice tail bleeding and stained with Giemsa's. These slides were used to determine pre-patent period and peak parasitaemias of the infected mice. Granules form of commercial anti-trypanosomal drug, berenil, from Hoechst Ag brand was obtained from Sigma-Aldrich (Germany). Every 2.36 g of the berenil granules contained 1.05 g 4,4'-diamidino diazoaminobenzine diaceturate. As described by Talakal *et al.* (2000), 3.5 mg/kg body weight is the best reservoir value of berenil for experimental mice. The granules were solubilised in 12.5 mL sterile $dH_2O$ as documented by Verma *et al.* (1976). While nerolidol (contained the mixture of ±40% of *cis*-3,7,11-trimethyl-1,6,10-dodecatrien-3-ol and ±55% of *trans*-3,7,11-trimethyl-1,6,10-dodecatrien-3-ol) was obtained from Merck (Germany). Every liter of the solution contained 880 g of nerolidol substance and at the concentration of 222.37 g/mol, the stock of nerolidol solution were prepared as described by Denise *et al*, (2004). *Allium sativum* in tablet form (Natural Factors Garlic, Canada) were used as the source for allicin treatment. Every tablet of 500 mg contained 750 µg allicin ($C_3H_5S_2OC_3H_5$) where this amount is similar as much as contained in 1500 mg of fresh garlic. Using two tablets from Natural Factors Garlic, an amount of 15 µg/ mL of allicin solution stock were prepared according to Ankri *et al*, (1997). Next, the dose at 0.1 ml of 8.8 µg/ml nerolidol solutions was given intraperitoneally while 0.1 ml of 15 µg/ml allicin solution was given orally to groups of mice according to the following regimes and groups: two negative and one positive control groups, three groups of pre-infection treatment (1 day, 3 days and 5 days before infection), one concurrent treatment group (5 minutes right after infection) and one post-infection treatment group (3 days after infection). The negative control groups either received 0.1 ml distilled water orally or lethal infection with $5.0 \times 10^3$ *T. evansi* per mouse given intraperitoneally without any treatment. Berenil at 0.01 ml of 3.5 mg/kg body weight was administrated into positive control mice which previously received *T. evansi* infection at 7 days before treatment. Blood samples were collected subsequently following the treatments and used to prepare for both the normal observations under light microscope using Leica CME model completed with Canon LA-DC58F digital camera and also for electron microscopy using Phillips XL30 and Leo 1450VP microscope. By using MINITAB version 16, the mean difference of parasitaemia level (%), survival time (day) and pre-patent time (day) of the mice were also determined by ANOVA test at $p<0.05$ significant level.

## RESULTS AND DISCUSSION

Generally, the results in Fig. 1, 2, 3 and 4 summarize the morphological changes of *T. evansi* in all groups of the experimental and berenil-treated mice. Beside these, the results also showed there were differences in survival times of all groups of the experimental mice which vary according to the test compounds used. As showed in Table 1 and Table 2, the results indicated that the earlier the test materials given before infection, the longer the pre-patent period observed and also the longer the mice survived. Overall, the allicin-treated group showed more convincing results, particularly for the KRA(a) group where interestingly, the mice survived up to more than 90 days post-treatment if treatment were given daily starting 7 days before infection and terminating at 30 days post infection. For all groups, the results showed that the treated-group mice always had higher peak of parasitaemias as compared to that of the peak parasitaemias in the control mice ($p<0.05$).

**Table 1** : Vital parameters observed from experimental mice in nerolidol-treated group.

| GROUP | | Nature of infection and treatment | PARAMETERS | | |
|---|---|---|---|---|---|
| Subgroup | Group Name | | Peak Parasitaemia (%)* | Pre-patent Period (days)* | Survival Time (days)* |
| Negative control | KKN(a) | *T. evansi* only without treatment | 38.67 ± 0.5 | 4.56 ± 0.3 | 8.41 ± 0.3 |
| | KKN(b) | Daily dose - $dH_2O$ given started at 7 days post-infection | 38.98 ± 0.2 | 5.47 ± 0.3 | 9.23 ± 0.2 |
| Positive control | KKP(a) | Single dose - Berenil given 7 days after | 18.79 ± 2.0 | 4.38 ± 0.2 | > 100 |
| Treatment with nerolidol (**) | KRN(a) | Daily dose - Started at 5 days pre-infection | 43.59 ± 1.2 | 21.48 ± 0.2 | 28.58 ± 0.2 |
| | KRN(b) | Daily dose - Started at 3 days pre-infection | 43.38 ± 1.7 | 15.42 ± 0.2 | 22.48 ± 0.2 |
| | KRN(c) | Daily dose - Started at 1 day pre-infection | 47.45 ± 1.6 | 8.45 ± 0.4 | 15.60 ± 0.2 |
| | KRN(d) | Daily dose - Started 5 minutes after infection | 43.77 ± 1.2 | 6.61 ± 0.2 | 13.65 ± 0.1 |
| | KRN(e) | Daily dose - Started 3 days post-infection | 36.02 ± 2.6 | 3.52 ± 0.2 | 8.54 ± 0.2 |

(*) Mean ± S.D.
(**) All daily dose given until the mice died or up to 30 days post infection.

**Table 2** : Vital parameters observed from experimental mice in allicin-treated group.

| GROUP | | Nature of infection and treatment | PARAMETERS | | |
|---|---|---|---|---|---|
| Subgroup | Group Name | | Peak Parasitaemia (%)* | Pre-patent Period (days)* | Survival Time (days)* |
| Negative control | KKN(c) | *T. evansi* only without treatment | 38.35 ± 1.7 | 4.56 ± 0.2 | 9.36 ± 0.1 |
| | KKN(d) | Daily dose - $dH_2O$ given started at 7 days post-infection | 39.74 ± 0.4 | 4.22 ± 0.1 | 9.11 ± 0.3 |
| Positive control | KKP(b) | Single dose - Berenil given 7 days after | 19.83 ± 1.2 | 4.56 ± 0.3 | > 100 |
| Treatment with allicin (**) | KRA(a) | Daily dose - Started at 7 days pre-infection | 47.09 ± 0.2 | 20.42 ± 0.3 | 96.58 ± 0.2 |
| | KRA(a) | Daily dose - Started at 5 days pre-infection | 42.81 ± 1.2 | 82.54 ± 0.2 | 91.67 ± 0.4 |
| | KRA(a) | Daily dose - Started at 3 days pre-infection | 46.24 ± 1.1 | 77.42 ± 0.2 | 86.48 ± 0.2 |
| | KRA(a) | Daily dose - Started at 1 day pre-infection | 42.51 ± 1.7 | 74.5 ± 0.2 | 83.43 ± 0.2 |
| | KRA(a) | Daily dose - Started 5 minutes after infection | 47.01 ± 2.3 | 8.60 ± 0.2 | 16.64 ± 0.3 |
| | KRA(a) | Daily dose - Started 3 days post-infection | 36.02 ± 2.6 | 3.52 ± 0.2 | 8.54 ± 0.2 |

(*) Mean ± S.D.
(**) All daily dose given until the mice died or up to 30 days post infection.

Fig. 1 shows the observation taken from the mice in group KRN(a). The pictures obviously revealed the normal morphology of *T. evansi* in the nerolidol-treated group as appeared after the 23rd day post-treatment, which gradually changed until the 25th day post-treatment where the parasites became stiff, lost their undulating membrane but still free flagellum remained intact. Total disfigurement was only observed at the 27th day post-treatment but it was too late to save the mice. This phenomenon can be related with an earlier report by Ogunlana *et al.* (1987) that stressed the ability of nerolidol to inhibit the synthesis of peptidoglycan molecule which is critically needed in constructing the parasite single cell bi-layer cell membrane such as *T. evansi*. Likewise, using *E. coli* as a model, Turi *et al.* (1997) also found that the capability of nerolidol to modify the hydrophobic characteristic on the surface of cell

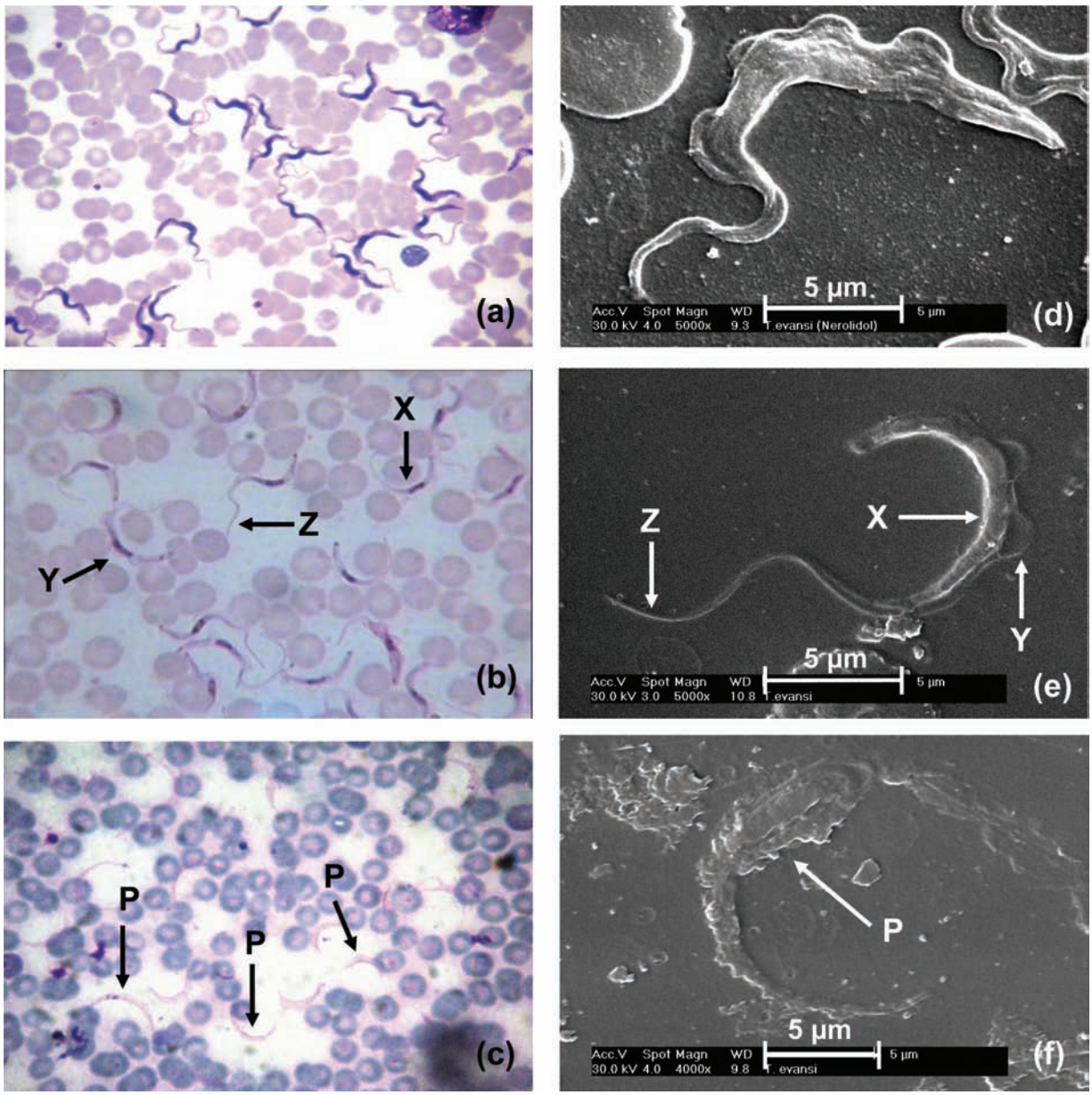


**Fig. 1.** The decrease of the parasite population and the morphological changes of *T. evansi* observed in light (×100 magnification) and electron (SEM at ×4000 – ×5000 magnification) microscope as appeared in nerolidol-treated mice blood from KRN(a) group, starting from 23 days post-treatment (a) and (d), 2 days after its pre-patent period and its continued gradually at 25 days post-treatment (b) and (e), where the parasites became stiff (X), lost their undulating membrane (Y) but the free flagella remained intact (Z) and at 27 days post-treatment (c) and (f) where total disfigurement (P) can be observed, but somehow it was too late to save the mice.

membrane might lead to the higher possibility of changing the original structure or morphology of cells in general.

Although parasites in the allicin-treated mice also showed some obvious morphological changes but the action was slow, unpredictable and not as drastic as that under berenil treatment. Gadually, the morphological changes only started to appear after about 18 days post-treatment and continued gradually up to the 90th day post treatment although 30 days the treatment was terminated after infection. The pictures in Fig.2 were taken from the mice in group KRA(a) where the parasites became more crescent in shape and lost most of the undulating membranes and cytoplasm. However, on the 94th day, the normal parasites reappeared in the blood

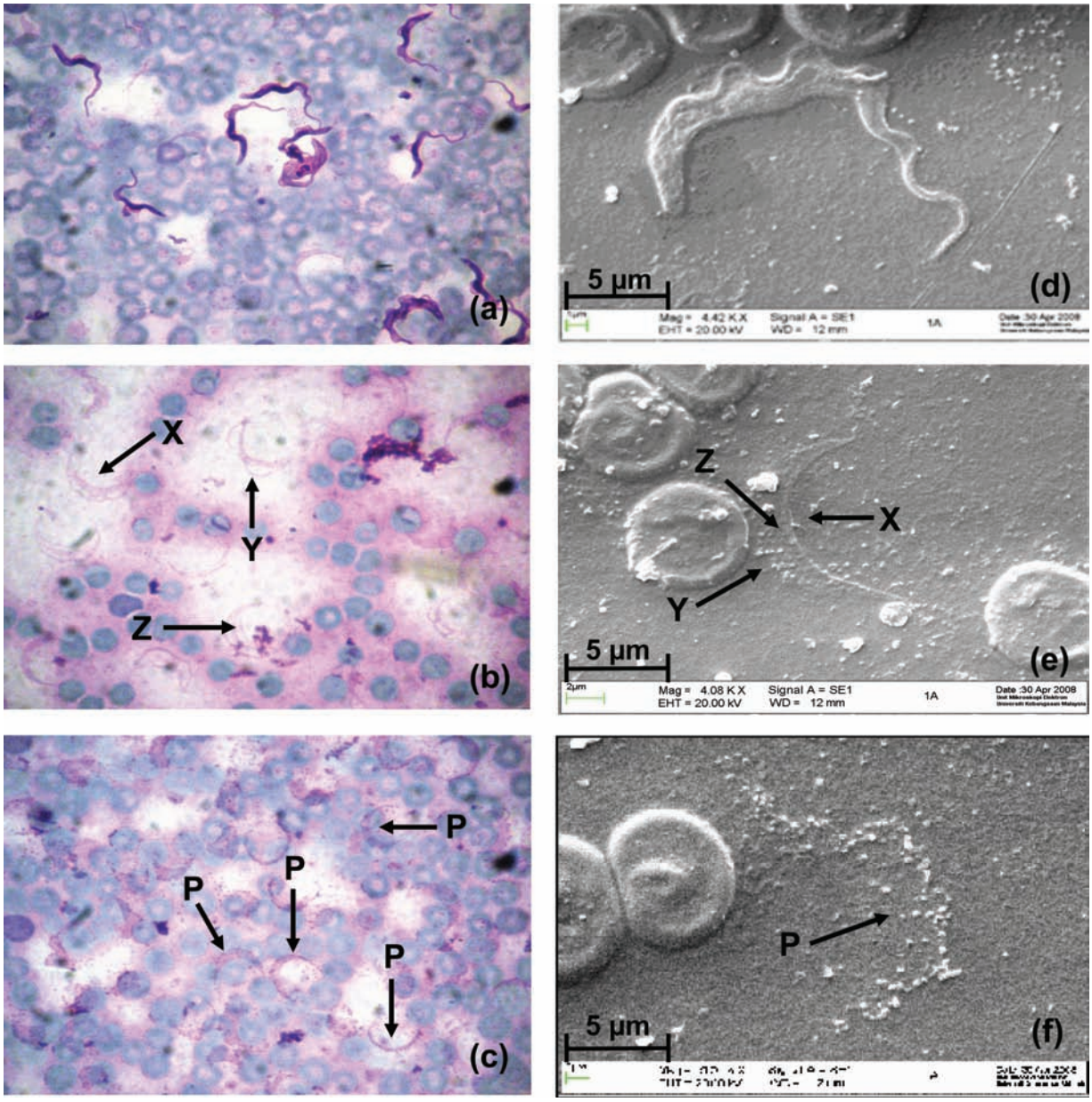


**Fig. 2.** The decrease of the parasite population and the morphological changes of *T. evansi* observed in light (×100 magnification) and electron (SEM at ×4000 – ×5000 magnification) microscope as appeared in allicin-treated mice blood from KRA(a) group at 30 days post-treatment (a) and (d) where allicin treatments were terminated on this day, and at 60 days later (b) and (e) where the parasites became more crescent in shape (X) and lost most of the undulating membranes (Y) and cytoplasm (Z) and at 93 days post-treatment (c) and (f), where total disfigurement (P) of some parasites was observed.

and maintained in the circulation together with the morphologically-changed *T. evansi* (Fig. 3) that may have caused the death of the mice in this group on day 96th. The anti-tripanosomal activities of allicin revealed in this study supports the earlier result that, allicin at 30 µg/ml very efficiently inhibited the growth of other protozoan parasites such as *Leishmania major* (Rabinkov *et al.*, 1998), the parasite belonging to the same phylum with *Trypanosoma.* Supporting this documentation, allicin and the condensation product of allicin, ajoene, which has a similar oxygenated sulfur group, has been shown to inhibit the proliferation of *Trypanosoma* sp., possibly by inhibition of phosphatidylcholine biosynthesis (Yabu *et al.*, 1998).

Morphological changes that occurred in *T. evansi* in berenil-treated mice appeared as soon as 2 – 3 hours post-treatment as shown in Fig. 4, where the parasites become stiffened and tapered at both ends. The most massive changes such as the loss of undulating membrane and fractured flagella appeared 4 hours post-treatment. By 5 – 6 hours post-treatment the parasites were totally squashed and disintegrated and cleared from the blood circulation. This observation happened in both the positive control of experimental mice, KKP(a), in nerolidol-treated group and KKP(b), in allicin-treated group. As a standard and the best drug in treating trypanosomiasis (Talakal *et al.*, 2000), the reason behind this finding is because berenil directly interacts by binding the parasite DNA at the A-T rich region (Clausen *et al.*, 1999), thus leading to the formation of hydrogen bonds whereby the vital DNA processes such as transcription and replication may be inhibited (Brown *et al.*, 1992).

The overall results of this study suggest that although the test materials studied were less effective to clear *T. evansi* from the blood circulation, the results imply that these bioactive compounds may have some anti-trypanosomatidal activities. These compounds might have acted sufficiently by slowing down or inhibiting the progress of the infection as to allow the mice to survive much longer. Thus, it is highly recommended that a further study is required to determine the gross effects of these bioactive compounds, particularly allicin, on the morphology of the parasite, as a proof of its action in this study. This is also suggested in order to elucidate the clear mechanism(s) of action of these compounds on the development of *T. evansi.*

## ACKNOWLEDGEMENTS

We appreciate the assistance provided by the School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia for making the study possible and also for the Kulliyyah of Allied Health Sciences, International Islamic University Malaysia for its full support to the authors.

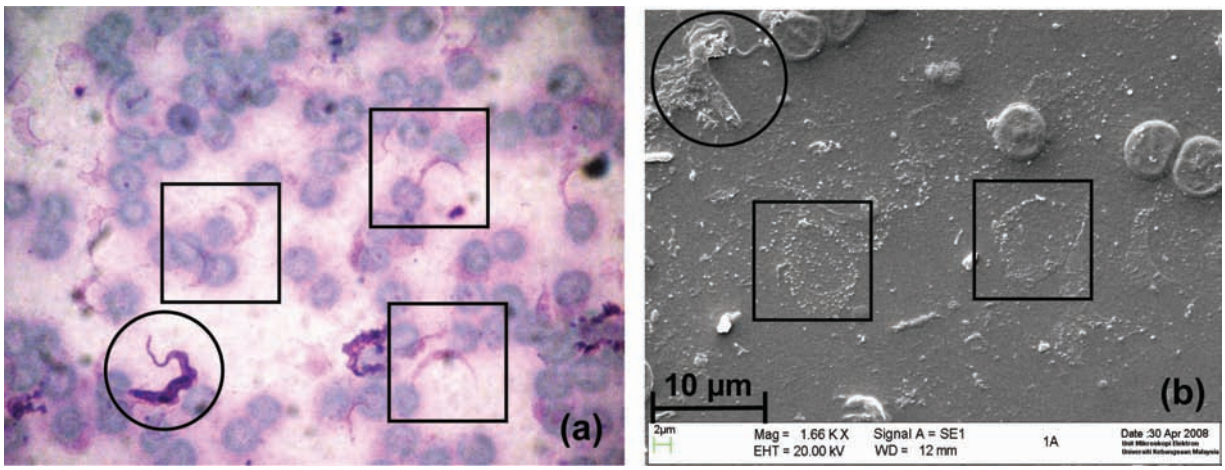


**Fig. 3.** (a) The mixture of normal shaped *T. evansi* (in circle) with crescent-shaped *T. evansi* (in square) in allicin-treated mice from KRA(a) group on day 94th post-treatment as observed in light microscope (×100 magnification) and (b) the mixture of normal morphology of *T. evansi* (in circle) with crescent-shape *T. evansi* (in square) as observed in electron microscope (SEM at ×1660 magnification).

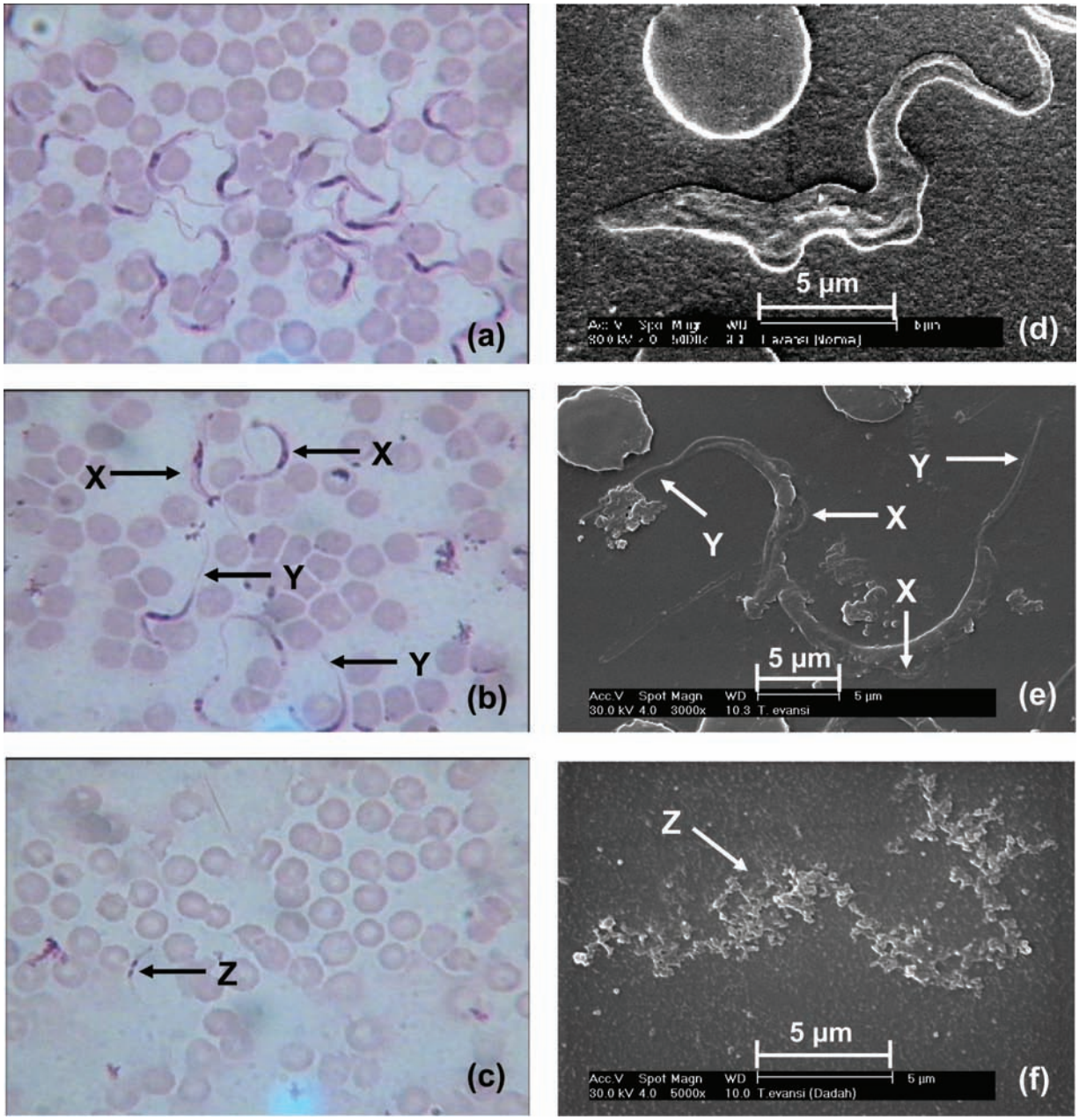


**Fig. 4.** The decrease of the parasite population and the morphological changes of *T. evansi* observed in light (×100 magnification) and electron (SEM at ×3000 – ×5000 magnification) microscope as appeared in berenil-treated mice blood (positive control) starting from 1 hour post-treatment (a) and (d), 3 hours post-treatment (b) and (e), where the most massive changes such as the loss of undulating membrane (X) and fractured flagella (Y) appeared, and 6 hours post-treatment (c) and (f), where the parasites were totally disintegrated (Z) and cleared from the blood circulation.

## REFERENCES

Ankri, S., Miron, T., Rabinikov, A., Wilchek, M. & Mirelman, D. 1997. Allicin from garlic strongly inhibits cysteine proteases and cytopathic effects of *Entamoeba histolytica*. *Antimicrobial Agents Chemotherapy*, **41**: 2286-2288.

Brown, D.G., Sanderson, M.R., Garman, E. & Neidle, S. 1992. Crystal structure of a berenil (CGCAAATTTGCG) complex: An example of drug-DNA recognition based on sequence-dependent structural features. *Journal of Molecular Biology,* **226**: 481-490.

Clausen, P.H., Waiswa, C., Katunguka-Rwakishaya, E., Scahres, G., Steuber, S. & Mehlitz, D. 1999. Polymerase chain reaction and DNA probe hybridization to assess the efficacy of diminazene treatment in *Trypanosoma brucei* infected cattle. *Parasitology Research*. **85**: 206-211.

Denise, C.A., Fabio, D.A., Alejendro, M.K. & Silvia, R.U. 2004. Antileishmanial activity of the terene nerolidol. Departmento de Parasitologia, Instituto de Ciencias Biomedicas, Universidade de Sao Paulo, Brazil.

Ogunlana, E.O., Hoeglund, S., Onawunmi, G. & Skoeld, O. 1987. Effects of lemongrass oil on the morphological characteristics and peptidoglycan synthesis of *Escherichia coli* cells. *International Microbiology*, **50**: 43-59.

Rabinkov, A., Wilchek, M. & Mirelman, D. 1998. The mode of action of allicin: Trapping of radicals and interaction with tiol containing proteins *Biochemstry Biophysics Acta,* **1379**: 233-244.

Talakal, T.S., Dwivedi, S.K. & Sharma, S.R. 2000. *In vitro* and *in vivo* antitrypanosoma potential of *nyctanthes arbor-tristis* leaves. *Pharmaceutical Biology*. **38(5):** 326-329.

Turi, M., Turi, E., Koljalg, S. & Mikelsaar, M. 1997. Influence of aqueous extracts of medicinal plants on surface hydrophobicity of *Escherichia coli* strains of different origin. *Acta Pathologica, Microbiologica et Immunologica Scandinavica,* **105**: 956-962.

Ventura, R.M., Carmen, S.A., Roberto, A.M., Nunest, V.L., Takeda, G.F. & Teixeira, M.M. G. 2000. Molecular and morphological studies of Brazillian *Trypanosoma evansi* stocks: the total absence of kDNA in trypanosome from both laboratory stocks and naturally infected domestic and wild mammals. *The Journal of Parasitology*. **86(6)**: 1289-1296.

Verma, B.B., Gautam, O.P. & Malik, P.D. 1976. *Trypanosoma evansi* : Therapeutic Effiacacy of Diaminazine Aceturate in Crossbred calves, *Bos taurus* and *Bos indicus*. *Experimental Parasitology*. **40**: 406-410.

Yabu, Y., Nose, M., Koided, T., Ohta, N. & Ogihara, Y. 1998. Antitrypanosal effects of tradisional Chinease herbal medicines on bloodstream forms of *Trypanosoma brucei rhodesiense in vitro*. *Southeast Asian Journal of Tropical Medicine and Public Health*. **29(3)**: 599-604.

Zainal-Abidin, B.A.H. 1992. Infections of *Trypanosoma evansi* in Malaysia. *Malaysian Applied Biology,* **2(10)**: 1-8.